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Antibody response to streptococcal cell wall antigens associated with experimental arthritis in rats

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SUMMARY

The antibody response to group A streptococcal cell wall components was measured in rats during the development of chronic, remittent experimental arthritis. The arthritis was induced by a single intraperitoneal injection of an aqueous suspension of group A streptococcal cell wall fragments and antibodies were measured by a radioactive antigen-binding assay. Antibodies in serum against both peptidoglycan and A polysaccharide reached maximum levels at 1 or 2 weeks and declined to preimmunization levels by day 63. The kinetics and magnitude of the antibody responses were similar in neonatally thymectomized and non-thymectomized rats. A relationship between chronic joint lesions and anti-peptidoglycan concentration in serum was indicated, since all rats which produced high levels of antibody developed severe chronic arthritis. However, 46% of the rats which produced very low levels of antibody also developed moderate to severe arthritis. There was no correlation between anti-A polysaccharide antibodies and joint disease, although the concentration of this antibody was 10- to 100-fold greater than the anti-peptidoglycan. We conclude that antibody can be a component in the pathogenesis of this experimental model of arthritis, but its role requires further elucidation.

INTRODUCTION

Rats given a single intraperitoneal injection of an aqueous suspension of group A streptococcal cell wall fragments develop a chronic, remittent, erosive polyarthritis of the fore and hind limbs (Cromartie *et al.*, 1977; Clark *et al.*, 1979). Joint inflammation is associated with the localization of cell wall fragments in the synovial and periarticular tissues, and cell wall antigens can be detected within these tissues by immunofluorescence for at least 180 days. The essential components of the purified cell walls are the covalently bound polymers of peptidoglycan and group-specific A polysaccharide.

The role of the immune response in this experimental arthritis is not clear. Since this disease develops in neonatally thymectomized rats and does not correlate with delayed hypersensitivity against peptidoglycan (Hunter *et al.*, 1980) it does not appear to be the result of cell-mediated immune mechanisms. The studies on the immune response have been extended by measuring the serum antibody levels against peptidoglycan and group A polysaccharide in rats given arthropathogenic doses of group A streptococcal cell wall fragments. This paper reports (i) the time-course of the antibody response to peptidoglycan and group A polysaccharide; (ii) the effect of cell wall dose

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and neonatal thymectomy on the antibody response to cell wall antigens; and (iii) the relationship of serum antibody concentrations to the development of experimental arthritis.

MATERIALS AND METHODS

Bacterial cells and cell walls. Detailed procedures have been described elsewhere (Hunter *et al.*, 1980; Cromartie *et al.*, 1977). Group A streptococci type 3, strain D-58 were grown in Todd–Hewitt broth (BBL Microbiology Systems, Cockeysville, Maryland) for 16 hr at 37°C. Cells were collected by centrifugation, washed three times with cold saline and disrupted in a Braun shaker. The cell walls were isolated by differential centrifugation, treated with trypsin and ribonuclease, washed, dialysed against distilled water and lyophilized.

Cell wall fragments. Purified group A streptococcal cell walls were suspended in phosphate-buffered saline (PBS) pH 7·2 and sonicated for 70 min in a Branson Sonifier (Model S125, Heat Systems Co., New York) at maximum power. The disrupted cell walls were then filtered through millipore filters (Millipore Corp., Bedford, Massachusetts) of 1·2- and 0·45-μm pore size. Preparations were tested for sterility by plating 0·1 ml onto sheep blood agar plates. The rhamnose content of the disrupted cell walls was measured by the method of Dische & Shettles (1948).

Cell wall peptidoglycan and A polysaccharide. Streptococcal group-specific polysaccharides were prepared from group A and group A-variant streptococci by extraction of purified cell walls by the formamide extraction method of Fuller (1938) as described by Krause & McCarty (1961). The peptidoglycan moiety was obtained by formamide extraction of group A-variant cell walls as described by Krause & McCarty (1961).

Induction of experimental arthritis. Outbred Sprague–Dawley rats were obtained from Zivic-Miller, Allison Park, Pennsylvania. Rats weighing approximately 100 g were used in all experiments. To induce arthritis, rats were given a single intraperitoneal injection of an aqueous suspension of sterile group A streptococcal cell wall fragments at doses of 60, 20 or 5 μg of rhamnose/g body weight. These doses are equivalent to 20, 6·6 or 1·66 mg of cell wall material per 100-g rat.

Quantitative precipitin analysis. Quantitative precipitin analysis was performed on hyperimmune rat antisera to determine the amount of antibody to A polysaccharide and peptidoglycan. These antisera were then diluted to contain known amounts of antibody and used to prepare standard curves in the antigen-binding assay. Rats were hyperimmunized with group A streptococcal vaccines as described by Greenblatt *et al.* (1971).

Tyrosylation and radioiodination of peptidoglycan and A polysaccharide. Before iodinating it was necessary to insert phenolic groups by first treating the polysaccharide or peptidoglycan with cyanogen bromide (Axen, Porath & Ernback, 1967) and then reacting the activated structures with tyramine as described by Gotschlich *et al.* (1972). Radioiodination was performed by a modification of the chloramine T method described by Gotschlich *et al.* (1972).

Antibody measurement. Antibodies specific for peptidoglycan and group A polysaccharide were measured by a radioactive antigen-binding assay. The methods described by Heymer *et al.* (1975a) for peptidoglycan, and Bernstein, Klapper & Krause (1975) for polysaccharide were adapted to rat serum. The double-isotope method of Gotschlich (1971) using ^{22}Na as a volume marker was used. Labelled polysaccharide bound to antibody was precipitated with 10% polyethylene glycol 6000. The per cent antigen bound was compared to a standard curve of known antibody concentrations as determined by quantitative precipitin analysis. A computer program was developed using a statistical analysis system (Barr *et al.*, 1976) to perform the calculations. Results are expressed as μg antibody/ml of serum.

Thymectomy. Neonatal thymectomy was performed within 24 hr of birth by a combination of blunt dissection and suction. Littermates were matched for thymectomy or sham-thymectomy and males and females were included in both groups. After the animals had been killed a histological evaluation of thymectomy was performed and animals with more than 10% thymus were not included in the data.

Statistical analysis. All data was indexed on computer cards and all statistical analyses including

Student's *t*-test, analysis of variance, correlation coefficients, linear regression analysis, lack-of-fit analysis and frequency distribution were performed using an IBM 360/75 computer and Statistical Analysis System (SAS) (Barr *et al.*, 1976). Unless otherwise noted all statistical analyses were performed using the logarithm of the antibody concentration.

Method of scoring arthritis. Arthritis was scored by a method similar to that of Wood, Pearson & Tanaka (1969) for scoring adjuvant arthritis. The severity of the arthritis was graded on a scale of 0–4 for each extremity with a maximum total possible score of 16. The scoring is based on the number of joints involved, severity and extent of erythema and oedema of the periarticular tissues and enlargement, distortion and ankylosis of the joints. The joints scored were those of each extremity distal to the knees and elbows. Joint lesions were also assessed by X-ray and scored by a radiographic technique (Clark *et al.*, 1979).

RESULTS

Kinetics of the antibody response in thymectomized and non-thymectomized rats

All animals were injected intraperitoneally with group A streptococcal cell wall fragments in a dose of 60 μg of rhamnose per g body weight. Serum was collected 3 days before injection and at 3, 7, 14, 30, 42 and 63 days after injection for measurement of antibodies against peptidoglycan and group-specific A polysaccharide. Rats were also examined clinically and radiographically for joint inflammation and bone changes.

The anti-A polysaccharide antibody in thymectomized and non-thymectomized rats is shown in Fig. 1. No antibodies were detected prior to immunization. The antibody concentration reached a

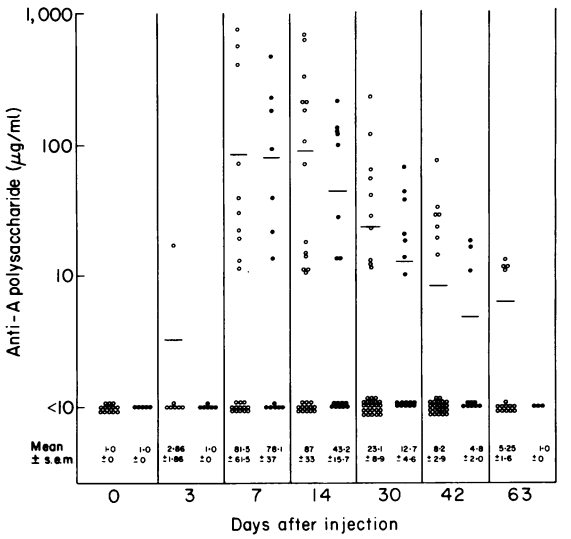

Fig. 1. Anti-A polysaccharide antibody concentration in the sera of neonatally thymectomized (○) and non-thymectomized (●) rats injected i.p. with group A streptococcal cell wall fragments (60 μg of rhamnose per g body weight). Bars indicate mean. Each point represents one rat.

peak at day 7 or day 14 and declined slowly toward preimmunization levels by day 63. The response of some individual thymectomized animals to the polysaccharide was higher than non-thymectomized rats, but the differences between the mean values of the two groups were not significant at any time interval. Though not shown here, unimmunized control rats examined over the same interval showed no measurable anti-A polysaccharide antibodies.

Serum anti-peptidoglycan antibody concentration in thymectomized and non-thymectomized

rats is shown in Fig. 2. Antibody was detectable in both groups 3 days after cell wall injection and reached peak levels between 7 to 14 days. Antibody levels then declined to preimmunization levels by day 63. Similar to the anti-A polysaccharide response, several thymectomized rats had higher anti-peptidoglycan antibody levels than non-thymectomized rats, but the differences between groups were not significant.

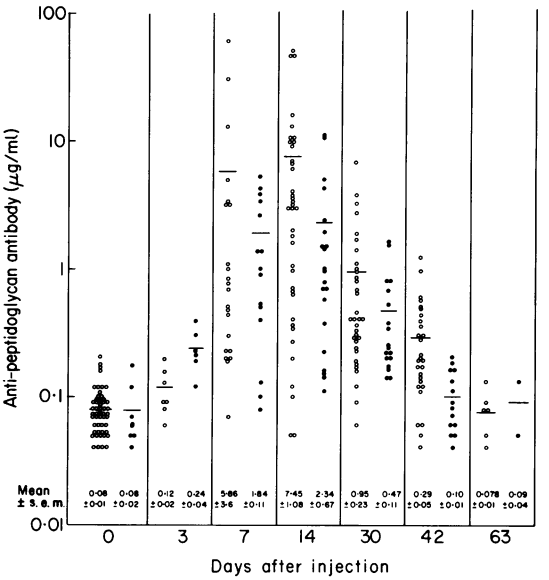

Fig. 2. Anti-peptidoglycan antibody concentration in the sera of neonatally thymectomized (o) and non-thymectomized (•) rats injected i.p. with group A streptococcal cell wall fragments (60 μg of rhamnose per g body weight).

Effect of cell wall dose on the antibody responses of thymectomized and non-thymectomized rats

The antibody responses to A polysaccharide and peptidoglycan were measured in thymectomized and non-thymectomized rats injected with cell wall fragments in doses of 60, 20 or 5 μg of rhamnose/g body weight. All groups reached peak antibody levels by 1 or 2 weeks.

The effects of dose and treatment (thymectomy and non-thymectomy), as well as interactions between dose and treatment were examined by analysis of variance. There were no significant quantitative differences in antibody to A polysaccharide over this range of arthritogenic doses of cell wall. When the maximum anti-A polysaccharide of non-thymectomized and thymectomized rats was compared, no significant difference between the two groups was observed ($P > 0{\cdot}22$). In addition, no interactions between dose and treatment were noted.

When the anti-peptidoglycan antibody levels of non-thymectomized rats were examined by analysis of variance, no dose-effect on the amount of antibody produced was observed over this limited range of cell wall concentration ($P > 0{\cdot}71$). In contrast, thymectomized rats showed a logarithmic increase in maximum antibody concentration with increasing dose ($P < 0{\cdot}005$). It was also observed that interactions between dose and treatment (thymectomy or non-thymectomy) were present ($P < 0{\cdot}02$). This means that there is a combined influence of the treatment (thymectomy) and dose of cell wall on antibody response over and above the effects of each of these factors considered separately.

Evaluation of the relationship between chronic joint lesion score and antibody concentration

The antibody and joint lesion data from thymectomized and non-thymectomized Sprague–Dawley rats, injected with group A streptococcal cell wall fragments in doses of 60 or 20 μg of rhamnose per g body weight, were examined by regression analysis. These groups were included in the analysis to provide a broad range of antibody and joint lesion responses. It can be seen in Fig. 3 that no

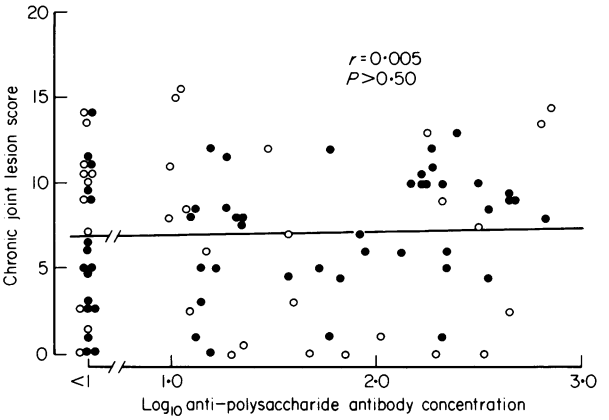

Fig. 3. Relationship between the maximum chronic joint score and the maximum concentration of anti-A polysaccharide measured in ninety-one rats. (•) Non-thymectomized, (○) thymectomized. This regression analysis includes thymectomized and non-thymectomized rats injected with cell wall fragments in a dose of 60 or 20 μg of rhamnose per g body weight.

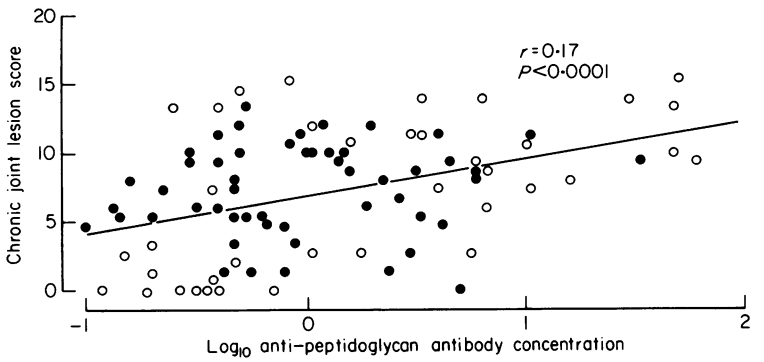

Fig. 4. Relationship between the maximum chronic joint score and the maximum concentration of anti-peptidoglycan. (•) Non-thymectomized, (○) thymectomized. (See legend to Fig. 3 for key.)

Table 1. Frequency distribution of chronic joint lesion score and anti-peptidoglycan concentration in serum

Maximum joint score	Anti-peptidoglycan (μg/ml)		
	0–1	1–10	10–100
< 1 (negligible)	12*	2	0
1–5 (moderate)	13	5	0
> 5 (severe)	22	27	10

* Number of rats.

relationship exists between the maximum antibody response to A polysaccharide and chronic joint lesion scores ($r=0{\cdot}005$). In a similar plot of the maximum antibody response to peptidoglycan versus the maximum chronic joint lesion score (Fig. 4) the correlation coefficient was low ($r=0{\cdot}17$) but significant ($P<0{\cdot}0001$). Since this indicates a relationship between joint lesion score and anti-peptidoglycan, a frequency distribution analysis of the data was performed (Table 1). All rats (10/91) with a relatively high concentration of anti-peptidoglycan antibody (>10 μg/ml of serum) developed severe chronic joint lesions. Amongst those rats with lower levels of antibody ($<10{\cdot}0$ μg/ml) a greater proportion developed moderate or negligible joint lesions. However, it is equally important to note that 46% (22/47) of the rats with very low antibody levels (0 to 1·0 μg/ml) also developed severe chronic joint lesions.

DISCUSSION

Intraperitoneal injection of rats with cell wall fragments isolated from group A streptococci induces an inflammation of the joints which reaches a peak in 3 to 5 days, depending upon the dose of cell wall. This acute phase recedes over the next 10 days and is followed by development of a chronic phase in which joint lesions recur in repeated cycles of remission and exacerbation over a period of 6 months (Cromartie *et al.*, 1977; Clark *et al.*, 1979).

Two points relevant to the clinical course of the experimental disease emerge from the kinetic study of antibody levels: (a) serum concentrations of both anti-A polysaccharide and anti-peptidoglycan antibodies are decreasing at the time the chronic phase of joint inflammation is developing, and the antibodies remain at background levels while the chronic remittent disease evolves; (b) the antibody levels are decreasing 2 to 3 weeks after cell wall injection in spite of the persistence of cell wall antigens within spleen, liver and joints for at least 180 days (Cromartie *et al.*, 1977). Jones, Amsbaugh & Prescott (1976) made similar observations in mice immunized with pneumococcal polysaccharide, an antigen which is also slowly degraded and persists in tissue for prolonged periods.

Both peptidoglycan and A polysaccharide would appear to be T-independent antigens since thymectomy does not reduce the antibody response to these antigens. In fact, some of the thymectomized rats exhibit higher antibody responses than non-thymectomized rats. The ability of peptidoglycan to activate complement (Greenblatt, Boackle & Schwab, 1978) and act as a B cell mitogen (Damais *et al.*, 1975) are consistent with properties of T-independent antigens. Group A polysaccharide, on the other hand, has been reported to be a T-dependent antigen in mice, at least when injected as a component of whole streptococcal cells (Braun, Kindred & Jacobson, 1972). Caution should be observed in defining these antigens as T-independent from the results presented here. Thymectomized rats do not seem to be as severely immune depressed as other thymectomized animals (Fisher & Fisher, 1965). The higher antibody response of some of the thymectomized rats may only reflect the early seeding of peripheral lymphoid organs by helper T cells and selective depletion of short-lived suppressor T cells (Kerbel & Eidinger, 1972). Furthermore, high doses of antigen, such as those used in these studies, have been reported to elicit a normal response in thymectomized animals (Bretscher, 1972).

A consistent feature of the immune response to group A polysaccharide and peptidoglycan is the variation in the magnitude of the antibody response between animals. Rabbits, mice and rats immunized intravenously with whole group A streptococci exhibit a similar variation. Even within inbred strains, variations in antibody concentrations from 100- to 1,000-fold have been observed (Eichmann, Braun & Krause, 1971; Braun *et al.*, 1972; Leslie & Carwile, 1973). This wide variation probably indicates that the antibody response to group A polysaccharide is under multigene control (Eichmann *et al.*, 1971; Briles, Krause & Davie, 1977). Similarly, anti-peptidoglycan antibody concentrations have been reported to vary by more than 150-fold (Heymer *et al.*, 1975b).

In a previous report an association between experimental joint disease and cell-mediated immunity against cell wall antigens could not be detected (Hunter *et al.*, 1980). The data presented here show no correlation between disease and serum levels of antibody against the cell wall polysaccharide. There is a trend toward association of higher serum levels of anti-peptidoglycan

with more severe joint disease, but further studies are required to determine if this is a causal relationship. Immune complexes, through the activation of complement and macrophages (Schorlemmer, Bitter-Suermann & Allison, 1977), can be a mechanism in the amplification of the experimental disease and in maintaining its chronic course. However, the peptidoglycan–polysaccharide cell wall structure can also activate the alternative complement pathway (Greenblatt *et al.*, 1978) and activate macrophages (Davies, Page & Allison, 1974; Smialowicz & Schwab, 1977) without the requirement of antibodies or lymphocytes. The essential component, common to either mechanism, is the persistent bacterial cell wall which provides the prolonged presence of antigenic and toxic peptidoglycan at the site of injury.

Studies on patients with rheumatoid arthritis also suggest a possible role for peptidoglycan and antibody in the pathogenesis of human disease. When compared to normal subjects, patients with rheumatoid arthritis have higher serum concentrations of anti-peptidoglycan antibodies (Schachenmayr, Heymer & Hafer Kamp, 1975; Pope *et al.*, 1979). Elevated levels of anti-peptidoglycan antibodies have also been observed in patients with juvenile rheumatoid arthritis and acute rheumatic fever (Heymer *et al.*, 1976; Rolieka & Massel, 1973).

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